REMARKS

Claims 13-21, 23-25, 27, 28 and 31 are pending in the application. Claim 21 has been amended to be consonant with the subject matter of claims in Group III, directed to methods of inhibiting the growth of tumor cells, as elected by Applicants in the Response to Restriction Requirement filed 16 April 2001. Support is found at least in the original claims and throughout the specification, for example pages 19-21. Claim 21 has been further amended as consistent with the specification (see page 20, line 1). Applicants respectfully request withdrawal of the Notice of Non-Responsive amendment and reconsideration of the outstanding rejections in light of the arguments presented to the Examiner in Applicants' response of 6 June 2003 and the following remarks.

In the Office Action of 5 December 2002, Claims 21, 23-25, 27, 28, and 31 had been rejected under 35 U.S.C. § 112, first paragraph for lack of enablement. Specifically, the Examiner's position was that the claimed methods lacked sufficient presumption of clinical utility.

Applicants submit for Examiner's review studies demonstrating the ability of anti-CTLA-4 antibodies directed against CTLA-4 extracellular domain and inhibitory of CTLA-4 signaling to inhibit the growth of tumor cells (Exhibit A: Hodi, F.S. et al., "Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients," *Proc. Natl. Acad. Sci. USA* 100(8):4712-4717 (2003); and Exhibit B: Phan, G.Q., et al., "Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma," *Proc. Natl. Acad. Sci. USA.* 100(14):8372-8377 (2003)). The references provide convincing evidence of the clinical effectiveness of anti-CTLA-4 antibodies in inhibiting tumor cell growth, especially when used with self antigen preparations. In Phan et al., vaccination with gp100 peptide alone had not elicited clinical tumor regression in any of the patients, but positive clinical results were seen when the self antigen was administered as an adjunct to anti-CTLA-4 antibody therapy (see page 8377, right column, first paragraph). Both of these references clearly evidence an autoreactive T cell response against normal and cancerous tissue expressing the self antigen utilized in the self antigen preparation. In view of the foregoing, withdrawal of the rejections under 35 U.S.C. § 112, first paragraph is respectfully requested. Withdrawal of other rejections in the

Office Action of 5 December 2002 is also requested in light of Applicants' response of 6 June 2003.

Applicants submit that the claims are in condition for allowance and an early notification of such is solicited. If the Examiner believes that prosecution of this case would benefit from a telephone interview, the Examiner is encouraged to call the undersigned at (415) 781-1989.

Respectfully submitted,
DORSEY & WHITNEY LLP

Dated: 12/08/03

By: _____
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Filed under 37 C.F.R. § 1.34(a)

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Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients

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Contributed by James P. Allison, February 19, 2003

A large number of cancer-associated gene products evoke immune recognition, but host reactions rarely impede disease progression. The weak immunogenicity of nascent tumors contributes to this failure in host defense. Therapeutic vaccines that enhance dendritic cell presentation of cancer antigens increase specific cellular and humoral responses, thereby effectuating tumor destruction in some cases. The attenuation of T cell activation by cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) further limits the potency of tumor immunity. In murine systems, the administration of antibodies that block CTLA-4 function inhibits the growth of moderately immunogenic tumors and, in combination with cancer vaccines, increases the rejection of poorly immunogenic tumors, albeit with a loss of tolerance to normal differentiation antigens. To gain a preliminary assessment of the biologic activity of antagonizing CTLA-4 function in humans, we infused a CTLA-4 blocking antibody (MDX-CTLA4) into nine previously immunized advanced cancer patients. MDX-CTLA4 stimulated extensive tumor necrosis with lymphocyte and granulocyte infiltrates in three of three metastatic melanoma patients and the reduction or stabilization of CA-125 levels in two of two metastatic ovarian carcinoma patients previously vaccinated with irradiated, autologous granulocyte–macrophage colony-stimulating factor-secreting tumor cells. MDX-CTLA4 did not elicit tumor necrosis in four of four metastatic melanoma patients previously immunized with defined melanosomal antigens. No serious toxicities directly attributable to the antibody were observed, although five of seven melanoma patients developed T cell reactivity to normal melanocytes. These findings suggest that CTLA-4 antibody blockade increases tumor immunity in some previously vaccinated cancer patients.

The formulation of genetic and biochemical strategies to identify cancer antigens yielded the unexpected discovery that tumor development frequently evokes immune recognition (1, 2). Cancer-associated gene products may stimulate T, B, and natural killer T (NKT) lymphocytes, natural killer cells, and phagocytes (3–7). Although the presence of brisk T cell infiltrates in human tumors is correlated with improved clinical outcomes, host responses in most cases are insufficient to inhibit disease progression (8–12).

One mechanism that may contribute to the failure of host defense is inadequate tumor antigen presentation (13). Cancer cells typically lack the expression of costimulatory molecules necessary to prime potent T lymphocyte responses directly, and dendritic cells infiltrating established tumors generally display limited maturation (14). Under these conditions, the induced tumor-reactive T cells manifest impaired functional capabilities. One strategy to ameliorate this defect in antigen presentation involves vaccination with irradiated tumor cells engineered to secrete granulocyte–macrophage colony-stimulating factor (GM-CSF) (15). Immunization elicits large numbers of activated $CD11b^+$ dendritic cells that express high levels of B7-1, B7-2, MHC II, and CD1d (16). These recruited cells efficiently phagocytose and process dying tumor cells, migrate to regional lymph nodes, and stimulate tumor-specific lymphocytes (17, 18). $CD4^+$ and $CD8^+$ T cells, CD1d-restricted invariant NKT cells, and antibodies mediate protective immunity (15, 16, 19, 20). A phase I clinical trial using retroviral-mediated gene transfer to engineer autologous GM-CSF-secreting melanoma cells established the ability of this vaccination scheme to enhance cancer immunity in metastatic melanoma patients (21). A second therapeutic strategy to improve tumor antigen presentation involves the loading of cancer antigens, in a variety of formulations, onto *ex vivo*-expanded dendritic cells (22). Several early-stage clinical trials have also demonstrated the ability of this vaccination scheme to increase tumor immunity (23–27).

Although a minority of patients achieved durable clinical responses in these studies, most eventually succumbed to progressive disease. One mechanism that may limit the therapeutic potency of cancer vaccines is the attenuation of T cell function by cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (28). Although the binding of B7-1 or B7-2 to CD28 provides an important costimulatory signal, the engagement of CTLA-4 by these ligands induces cell cycle arrest and diminished cytokine production (29–31). The development of a lethal lymphoproliferative disorder in young CTLA-4-deficient mice illuminates the pivotal role of CTLA-4 in immune homeostasis (32, 33). As $CD4^+$, but not $CD8^+$, T cell depletion reduces the autoimmune disease in these mice, the activities of CTLA-4 are essential for normal helper T cell regulation (34).

In contrast to the severe pathology characteristic of CTLA-4-deficient mice, transient CTLA-4 antibody blockade enhances antigen-specific T cell responses with limited toxicities. The injection of anti-CTLA-4 antibodies stimulates the rejection of moderately immunogenic murine tumors, and this activity may be potentiated with chemotherapy (35–38). Although CTLA-4 antibody blockade alone elicits minimal effects against poorly immunogenic tumors, concurrent vaccination with irradiated, GM-CSF-secreting tumor cells is highly efficacious in the B16 melanoma, SM1 breast carcinoma, and transgenic adenocarcinoma of the mouse prostate (TRAMP) carcinoma models (39–41). Cytotoxic T cells are critical for tumor destruction, but the augmented anticancer response may be associated with the

Abbreviations: CTLA-4, cytotoxic T lymphocyte-associated antigen 4; GM-CSF, granulocyte–macrophage colony-stimulating factor.

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EXHIBIT A

Table 1. Patient characteristics

Patient	Age	Diagnosis	Treatment 1	Recurrence	Treatment 2	Treatment 3	Treatment 4	MDX-CTLA-4	Metastases
1	43	Skin (MM) 1995	Surgery	Parotid, lung 1998	GVAX 7/98–2/99	Chemo 2/99–9/99	PS341 4/00–6/00	1/01	CNS, lung, abdomen, skin
2	56	LN (MM) 1992	Surgery, α-IFN	SC, perirenal, LN, 1998	GVAX 6/98–11/99	—	—	1/01	LN, bone, perirenal
3	43	LN (MM) 1997	Surgery, α-IFN	LN, intestine 1998	Surgery 12/98	DC 4/00–9/00	—	11/00	LN
4	58	Skin (MM) 1998	Surgery, XRT	Skin, lung 2000	DC 5/00–8/00	XRT 8/00	—	1/01	CNS, lung
5	49	Skin, LN (MM) 1998	Surgery, α-IFN	SC 2000	DC 11/00–2/00	—	—	4/01	SC, lung, liver, LN
6	52	SC (MM) 1997	Surgery, GM2	LN, lung 2000	gp100 12/00–3/01	Chemo 4/01–6/01	—	7/01	Lung, LN
7	31	Skin (MM) 1994	Surgery	LN, lung, SC, CNS, 1996	Surgery, chemo, 8/96	GVAX 7/98–10/98	Chemo, XRT 1/99–6/99, 2/01–10/01	9/02	SC
8	38	Pelvis (OV) 1998	Surgery, chemo	Lung, pelvis 7/99	Chemo 8/99–12/99	MUC-1 4/00–6/00	Chemo, GVAX, CI-1033, 6/00, 4/01, 2/02	6/02 10/02	Lung, pelvis, retroperitone
9	65	PER (OV) 1999	Chemo	PER 2001	Chemo 9/01–12/01	Chemo 2/02–5/02	GVAX 6/02–8/02	9/02	PER

MM, malignant melanoma; OV, ovarian carcinoma; SC, subcutaneous tissue; LN, lymph node; PER, peritoneum; GM2, ganglioside + QS-21; GVAX, irradiated, autologous tumor cells engineered to secrete GM-CSF; DC, dendritic cells engineered to express gp100 and MART-1; gp100, peptide plus IL-2; MUC-1, MUC-1 conjugated to KLH plus QS-21; XRT, radiation therapy; PS341, proteosome inhibitor; CI-1033, epidermal growth factor kinase inhibitor.

loss of tolerance to normal differentiation antigens, culminating in autoimmune vitiligo or prostatitis (42).

To gain a preliminary assessment of the biologic activity of antagonizing CTLA-4 function in humans, we administered the CTLA-4 blocking antibody MDX-CTLA-4 to nine previously vaccinated metastatic melanoma or ovarian carcinoma patients.

Materials and Methods

Clinical Protocols. The phase I studies of vaccination with irradiated, autologous melanoma or ovarian carcinoma cells engineered to secrete GM-CSF by adenoviral-mediated gene transfer will be reported elsewhere. The phase I study of vaccination with autologous dendritic cells engineered to express gp100 and MART-1 by adenoviral-mediated gene transfer will also be presented separately. The studies of vaccination with GM2 ganglioside admixed with QS-21 and immunization with a modified gp100 peptide plus IL-2 have been described (43, 44).

Patients 1–6 were enrolled in the Medarex-sponsored phase I trial MDX-CTLA-4-02 that was approved by the Dana–Farber Partners Cancer Care Institutional Review Board. Patients were eligible for this study if they had surgically unresectable stage III or stage IV malignant melanoma, disease progression, a life expectancy of at least 12 weeks, adequate end organ function, stable analgesic therapy, and a Karnofsky performance status of at least 60%. Patients were excluded if they used corticosteroids or had a second malignancy other than treated nonmelanoma skin cancer or superficial bladder cancer, autoimmune disease, active infection, or hypersensitivity to kanamycin. Patients 7–9 were treated on a Dana–Farber Partners Cancer Care-initiated trial of MDX-CTLA-4 infusion for metastatic melanoma, metastatic ovarian carcinoma, metastatic nonsmall cell lung carcinoma, or acute myelogenous leukemia patients previously vaccinated with irradiated, autologous, GM-CSF-secreting tumor cells. (The entire study, which will involve 16 patients, will be reported separately after its completion.) All patients provided written informed consent before enrollment in each clinical trial.

MDX-CTLA-4 is a human IgG1 antibody obtained from transgenic HuMAb mice, strain HC2/Kco7 (Medarex), immunized with the extracellular domain of CTLA-4. The antibody blocks binding of B7-1 Ig and B7-2 Ig to CTLA-4. MDX-CTLA-4 was drawn through a 0.22-μm filter and diluted in normal saline to a concentration of 2.5 mg/ml for administration. A test dose of 0.2 mg in 10 ml of normal saline was infused i.v. over 10 min to identify potential hypersensitivity reactions. The remainder of the 3 mg/kg MDX-CTLA-4 single dose was then delivered over 90 min with a volumetric pump. Patients were seen three times daily, four times weekly, and at monthly intervals thereafter for routine clinical, laboratory, and radiographic evaluation.

Pathology. Tissues were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Immunohistochemistry was performed by using standard techniques with monoclonal antibodies to CD4, CD8, CD20, and Ig-κ.

Results

Patient Characteristics. Seven metastatic melanoma and two ovarian carcinoma patients received MDX-CTLA-4 therapy between November 2000 and October 2002 (Table 1). The melanoma patients were all males with a median age of 49 years (range of 31–58 years). The average interval between the initial diagnosis of melanoma and study entry was 5 years (range of 3–9 years). Four patients received adjuvant therapies for early-stage disease (α-IFN, $n = 3$; vaccination with GM2 ganglioside admixed with QS-21, $n = 1$; radiation, $n = 1$). Nonimmunologic treatments for metastatic disease before enrollment were surgery ($n = 4$), radiation therapy ($n = 2$), chemotherapy ($n = 3$), and proteasome inhibitor ($n = 1$). The two ovarian carcinoma patients received multiple chemotherapies for relapsing disease throughout the 3–4 years before study enrollment.

All nine subjects participated in phase I vaccine studies for metastatic disease before entry into the MDX-CTLA-4 trial. Three melanoma and both ovarian carcinoma patients were immunized with irradiated, autologous tumor cells engineered to

Table 2. Biologic activity of CTLA-4 antibody blockade

Patient	Prior vaccine	Autoantibodies	Δ Neut	Melanocyte reactivity	Antitumor effects
1	GVAX	None	2	Skin	Extensive hemorrhagic necrosis with granulocytes and lymphocytes
2	GVAX	ANA 1:160	4	Skin retina	Extensive necrosis with granulocytes and lymphocytes; vasculopathy
3	DC	ANA 1:80, α-TG 212	3	Skin	$CD8^+$ T cell infiltrate; no tumor necrosis
4	DC	RF 28	0	Skin	$CD8^+$ T cell infiltrate; no tumor necrosis
5	DC	None	2.5	Skin	No tumor necrosis; absent infiltrate
6	GM2, gp100	None	0	No	Not done
7	GVAX	None	0	No	Extensive necrosis with granulocytes and lymphocytes; vasculopathy
8	MUC-1, GVAX	None	0	No	CA-125 reduction
9	GVAX	ANA 1:160	0	No	CA-125 stabilization

GVAX, irradiated, autologous tumor cells engineered to secrete GM-CSF; DC, dendritic cells engineered to express gp100 and MART-1; gp100, peptide plus IL-2; MUC-1, MUC-1 conjugated to KLH plus QS-21; ANA, antinuclear antibodies; α-TG, antithyroglobulin antibodies; RF, rheumatoid factor; Δ Neut, fold increase in neutrophils.

secrete GM-CSF by adenoviral-mediated gene transfer (patient 8 also received a MUC-1 vaccine). Three melanoma patients were immunized with autologous dendritic cells engineered to express gp100 and MART-1 by adenoviral-mediated gene transfer. One melanoma patient was vaccinated with a modified gp100 peptide and high-dose IL-2.

MDX-CTLA-4 Toxicities. A single dose of the human MDX-CTLA-4 antibody (3 mg/kg) was administered i.v. over 1.5 h. There was one acute hypersensitivity reaction characterized by mild hypotension and nausea during the infusion; this was easily controlled with antihistamines, and the treatment was completed uneventfully. Five patients developed transient grade 1–2 constitutional symptoms consisting of myalgias, arthralgias, anorexia, fatigue, nasal congestion, and nonproductive cough 2–7 days after the infusion; in one case, the syndrome recurred intermittently for several months. One patient with hepatic metastases manifested a transient grade 3 liver function test abnormality. Otherwise, there were no significant renal, pulmonary, cardiac, hematologic, gastrointestinal, or neurologic toxicities directly attributable to the antibody.

Autoimmune Reactions. MDX-CTLA-4 stimulated low titers of autoantibodies that persisted for 1–2 months without clinical evidence of autoimmune disease in four patients (Table 2). These included antinuclear antibodies (speckled pattern at 1:80 or 1:160 dilutions), antithyroglobulin antibodies (212, normal <60), and rheumatoid factors (28, normal <15). Four subjects mounted short-lived (24 h to 2 weeks) increases in circulating neutrophil counts (2- to 4-fold elevations).

All of the melanoma patients developed an asymptomatic, grade 1 reticular and erythematous rash on the trunk and extremities between 3 days and 3 weeks after MDX-CTLA-4 infusion (Fig. 1*A*). Punch biopsies of the skin in five of the seven subjects revealed prominent peri-vascular T cell infiltrates in the superficial dermis that extended into the epidermis (Fig. 1*B*). $CD4^+$ and $CD8^+$ T cells were found apposed to dying melanocytes in these sections (Fig. 1 *C* and *D*), although vitiligo was not clinically evident. Mild, focal hypopigmentation of the retinal pigmented epithelium was also detected by ophthalmologic examination in one patient, but this was not associated with a change in visual acuity. One ovarian carcinoma patient (no. 8) developed a transient erythematous rash on the face and trunk 2 weeks after infusion. Skin biopsy demonstrated perivascular T cell infiltrates in the superficial dermis, but no host reactivity toward melanocytes (not shown). Changes similar to these may be observed in hypersensitivity responses or some connective tissue diseases.

Antitumor Effects. MDX-CTLA-4 elicited extensive tumor necrosis with immune infiltrates in the three melanoma patients previously vaccinated with irradiated, autologous GM-CSF-secreting tumor cells. Patient 1 harbored metastases in the CNS, lungs, abdomen, and soft tissues at study entry. One month after MDX-CTLA-4 administration, a distinct change in clinical status was noted. A s.c. nodule became acutely inflamed (Fig. 2*A*), and facial twitching, slurred speech, impaired coordination, and weakness developed shortly thereafter. Magnetic resonance imaging revealed an increase in the gadolinium uptake of multiple brain lesions, suggesting an alteration in blood flow. Several cord compressions were detected in the cervical and thoracic spine, and large visceral metastases were present in the abdomen and lung. The patient deteriorated rapidly and died 6 days later, likely from CNS disease. Unexpectedly, marked hemorrhagic tumor necrosis was found at gross autopsy in numerous brain, epidural, and visceral metastases (Fig. 2 *B* and *C*). Histopathologic examination disclosed extensive tumor destruction (at least 90%) with hemorrhage (Fig. 2 *D* and *E*). While a rim of viable tumor cells persisted in each lesion, this was accompanied by a granulocyte and lymphocyte reaction (Fig. 2*F*).

Patient 2 manifested recurrent episodes of grade 2 constitutional symptoms beginning 1 month after MDX-CTLA-4 infusion. A biopsy of a mediastinal mass revealed extensive tumor necrosis with lymphocyte and granulocyte infiltrates (Fig. 3 *A*

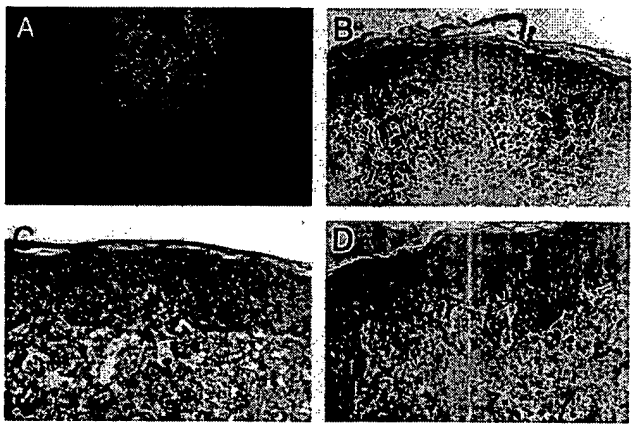

Fig. 1. MDX-CTLA-4 stimulated melanocyte immune recognition. (*A*) Reticular erythematous rash. (*B*) Perivascular lymphocyte infiltrate extending into epidermis with interface dermatitis. (*C*) $CD4^+$ T cells apposed to dying melanocytes. (*D*) $CD8^+$ T cells apposed to dying melanocytes. (Magnification: ×125, *B*; ×250, *C* and *D*.)

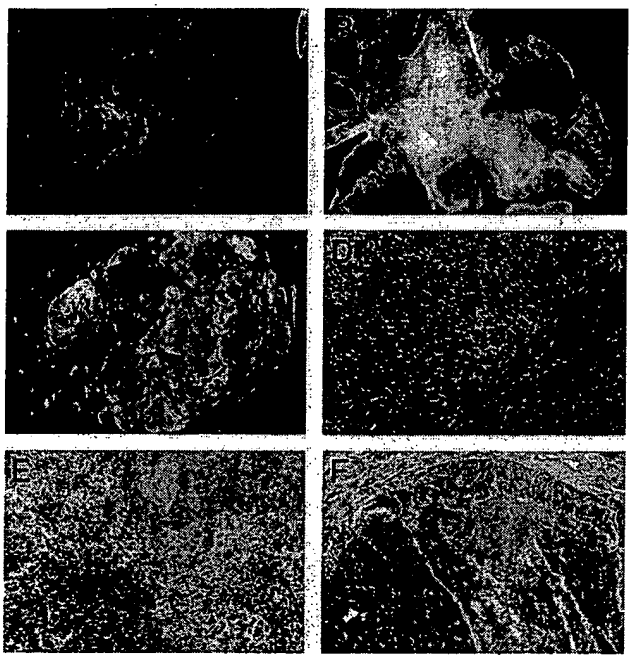

Fig. 2. MDX-CTLA-4 induced hemorrhagic tumor necrosis in vaccinated patient 1. (*A*) Inflamed s.c. nodule. (*B*) Necrotic brain metastasis. (*C*) Necrotic lung metastasis. (*D* and *E*) Hemorrhagic tumor necrosis. (*F*) Rim of viable tumor with granulocytes and lymphocytes. (Magnification: ×250, *D–F*.)

and *B*). Neutrophils were more prominent than eosinophils in the reaction. Immunohistochemistry disclosed the presence of $CD4^+$ and $CD8^+$ T cells and $CD20^+$ B lymphocytes producing Ig (Fig. 3 *C–E*). The lesion was completely resected 2 months later, and pathologic analysis demonstrated dense fibrosis, extensive necrosis, and an ongoing lymphocyte and granulocyte response (not shown). A vasculopathy characterized by a circumferential lymphoid infiltrate in the wall of an occluded blood vessel was also noted (Fig. 3*F*); tumor necrosis appeared spatially related to the vessel damage. Three months afterward, a zygomatic arch metastasis recurred. Chemotherapy with cisplatin and dacarbazine induced substantial tumor regression that persisted for 18 months.

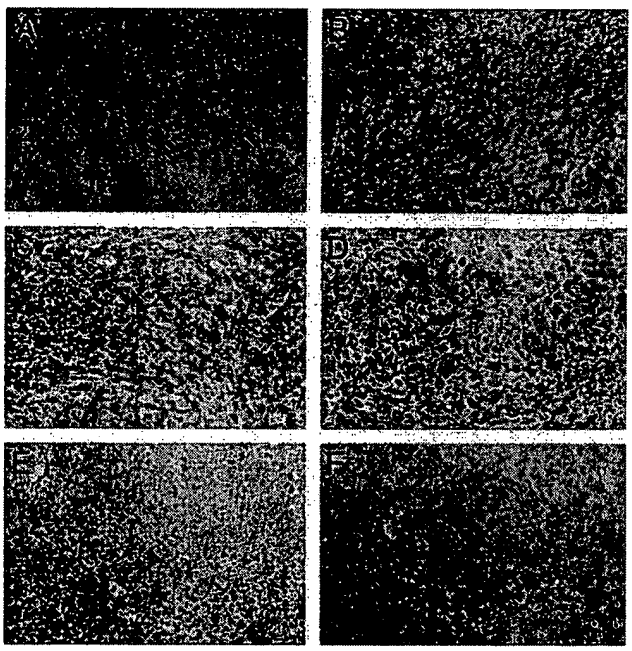

Fig. 3. MDX-CTLA-4 induced extensive tumor necrosis in vaccinated patient 2. (*A* and *B*) Tumor necrosis with granulocytes and lymphocytes. (*C*) $CD4^+$ T cells. (*D*) $CD8^+$ T cells. (*E*) $CD20^+$ B cells. (*F*) Vasculopathy with perivascular and intramural lymphoid infiltrates associated with luminal thrombosis. (Magnification: ×125, *A*; ×250, *B–F*.)

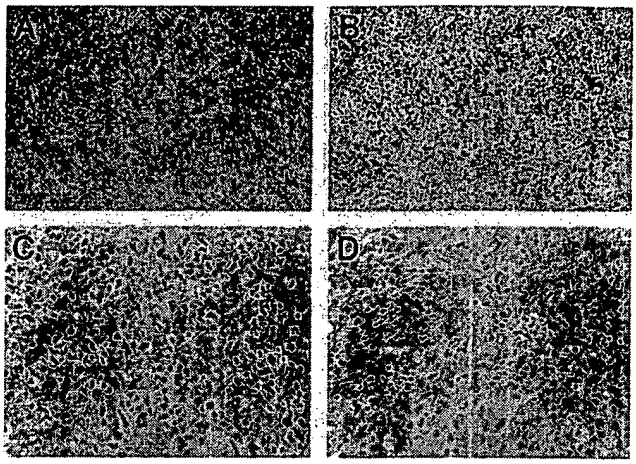

Fig. 4. MDX-CTLA-4 induced $CD8^+$ T cell infiltrates but no tumor necrosis in vaccinated patients 3 and 4. (*A*) Lymphocyte infiltrate without tumor destruction. (*B*) $CD8^+$ T cells. (*C*) $CD4^+$ T cells. (*D*) $CD20^+$ B cells. (Magnification: ×250, *A* and *B*; ×500, *C* and *D*.)

Patient 7 developed inflammation in a large s.c. mass 3 weeks after MDX-CTLA-4 infusion. The lesion was excised at 2 months, and pathologic examination similarly revealed extensive tumor necrosis and fibrosis with lymphocyte and granulocyte infiltrates. Moreover, perivascular lymphoid aggregates and infiltration of the vessel wall associated with thrombosis was again observed (data not shown).

CTLA-4 antibody blockade evoked less significant antitumor effects in the four melanoma patients previously immunized with defined melanosomal antigens. Patient 3 underwent a resection of an enlarging mediastinal mass 7 months after MDX-CTLA-4 infusion. Pathologic study revealed a dense lymphocyte infiltrate without tumor necrosis (Fig. 4*A*). Immunohistochemistry disclosed the presence of $CD8^+$ T, but not $CD4^+$ T or $CD20^+$ B, cells (Fig. 4 *B–D*). Although a brief period of stable disease followed treatment with cisplatin and dacarbazine, brain metastases developed shortly thereafter. Patient 4 showed a comparable $CD8^+$ T cell infiltrate without tumor necrosis in a lymph node metastasis resected 2 months after antibody administration. Subsequent chemotherapy similarly did not achieve a response, and the patient died 10 months after study entry. Patient 5 failed to develop lymphoid infiltrates or tumor necrosis in a s.c. metastasis resected 2 months after MDX-CTLA-4 administration (data not shown); follow-up chemotherapy was ineffective. Lastly, although patient 6 did not undergo a biopsy after MDX-CTLA-4 infusion, his clinical course was characterized by steady tumor progression that proved unresponsive to chemotherapy.

Although tumor biopsies could not be obtained in the two ovarian carcinoma patients after MDX-CTLA-4 infusion, the antibody elicited clear changes in blood CA-125 levels. This glycoprotein antigen is shed from the surface of ovarian carcinoma cells, thereby serving as a useful marker of disease status (45). Patient 8 showed a 43% reduction in CA-125 values (from 230 to 132) beginning 2 months after antibody infusion; although this response was not maintained, a second infusion of MDX-CTLA-4 stabilized CA-125 levels for 2 months (Fig. 5*A*). Patient

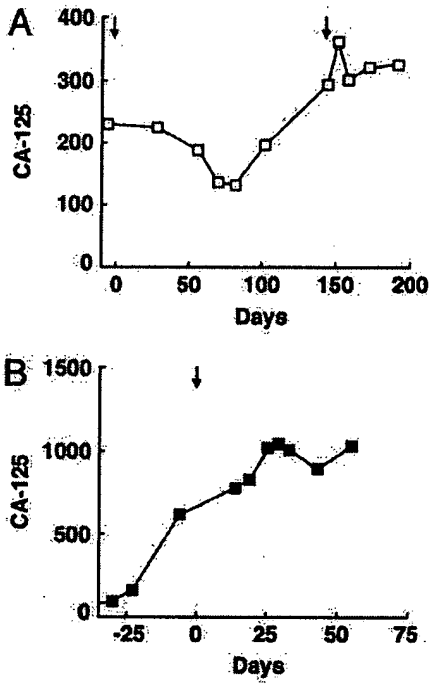

Fig. 5. MDX-CTLA-4 induced alterations in the circulating ovarian carcinoma tumor marker CA-125. (*A*) Patient 8. (*B*) Patient 9. Arrows indicate MDX-CTLA-4 infusions.

9 achieved a plateau in CA-125 values 1 month after antibody infusion (concomitant with a reduction in ascites and pain), despite rapidly rising levels before treatment (Fig. 5*B*).

Discussion

This phase I clinical investigation was undertaken in an effort to obtain a preliminary assessment of the biologic activity and toxicity of MDX-CTLA-4 in previously vaccinated metastatic melanoma and ovarian carcinoma patients. The study was motivated by compelling preclinical data indicating that the combination of CTLA-4 antibody blockade and cancer vaccination stimulated greater levels of antitumor immunity than either approach alone. Because the combination treatment also provoked a loss of tolerance to normal differentiation antigens, the risk of serious toxicities to patients was of some concern. Hence, we initially elected to administer CTLA-4 antibody blockade to previously vaccinated cancer patients.

Our initial results suggest that a single infusion of MDX-CTLA-4 may be safely delivered in this clinical setting. The generation of low titers of autoantibodies shows that the therapy may at least partially compromise systemic tolerance, but no evidence for autoimmune disease was noted. The melanoma patients developed a reticular and erythematous rash caused by perivascular lymphoid aggregates in the superficial dermis that extended into the epidermis. Both $CD4^+$ and $CD8^+$ T cells were juxtaposed with dying melanocytes in the skin, and one patient manifested focal hypopigmentation of the retinal pigmented epithelium as well. Although these findings demonstrate a loss of tolerance to melanocyte differentiation antigens, neither vitiligo nor alterations in visual acuity ensued, likely reflecting incomplete melanocyte destruction. Although the preclinical studies delineated a critical role for $CD8^+$ T cells in mediating depigmentation (42), the data presented here suggest that $CD4^+$ T cells may also contribute to melanocyte recognition.

MDX-CTLA-4 elicited antitumor effects in five of five patients previously immunized with irradiated, autologous GM-CSF-secreting tumor cells, whereas minimal tumor destruction was noted in the four patients previously immunized with defined melanosomal antigens. These preliminary findings raise the possibility that specific characteristics of preexisting tumor immunity may influence the response to subsequent CTLA-4 antibody blockade. The results may also be consistent with experiments showing that CTLA-4 antibody blockade elicits potent antitumor effects against moderately immunogenic, but not poorly immunogenic, murine tumors (35–41).

The mechanisms underlying the ability of CTLA-4 antibody blockade to increase tumor immunity remain to be clarified. Recent investigations reveal that CTLA-4 traffics to the immunologic synapse in response to T cell activation, thereby delivering an attenuating signal (46, 47). In immunized patients, tumor-reactive memory or effector T cells encountering antigen-loaded dendritic cells in the periphery or secondary lymphoid tissues may be the targets for CTLA-4 antibody blockade. Alternatively, MDX-CTLA-4 may modulate the activities of regulatory T cells that constitutively express surface CTLA-4 (48). In either or both cases, the relative importance of CTLA-4 blockade in augmenting effector T cell function versus modifying the affinity and/or breadth of T cell tumor recognition remains to be delineated.

Pathologic analysis of the metastases resected after MDX-CTLA-4 infusion disclosed several pathways that contributed to tumor destruction. The autopsy of patient 1 revealed striking hemorrhagic necrosis in all of the lesions examined. Tumor blood vessels were severely damaged in these masses, resulting in extensive ischemic necrosis and some bleeding. A rim of viable tumor was still present, but it was infiltrated with granulocytes and lymphocytes. Similar pathologic features were originally described in response to Coley's toxins (49), and tumor necrosis factor was later identified as one mediator of the reaction (50). Although the mechanisms underlying hemorrhagic necrosis are not yet fully defined, both soluble factors and leukocytes participate in vessel destruction (51). Indeed, a striking circumferential lymphoid infiltrate was detected in occluded tumor blood vessels in patients 2 and 7. As this mechanism of tumor destruction does not involve a rapid reduction in tumor volume, MDX-CTLA-4 may not prove clinically useful, however, in the setting of large CNS metastases.

An unexpected finding was the prominent effect of CTLA-4 antibody blockade on neutrophil responses. MDX-CTLA-4 induced significant increases in circulating neutrophils, and robust neutrophil infiltrates were associated with tumor necrosis. A primary role for neutrophils in mediating tumor destruction was previously suggested by experiments characterizing the host reaction to tumor cells engineered to secrete G-CSF (52). As T cells from CTLA-4-deficient mice show enhanced secretion of multiple cytokines including GM-CSF (33, 53), the recruitment of neutrophils may be secondary to T cell activation.

The serial biopsies of the mediastinal mass from patient 2 suggested the possibility of the evolution of a coordinated cellular and humoral antitumor response. Immunohistochemistry disclosed $CD4^+$ and $CD8^+$ T cells and $CD20^+$ B cells producing Ig. Although the preclinical studies underscored the importance of cytotoxic T cells in effectuating tumor destruction (42), these results suggest that a broader lymphocyte reaction may be involved. Indeed, $CD8^+$ T cells (without $CD4^+$ and $CD20^+$ lymphocytes) were detected in the metastases of two patients previously immunized with melanosomal antigens; however, neither lesion manifested tumor necrosis. More detailed investigations of the functions of helper T and B cells in the antitumor effects of CTLA-4 antibody blockade are warranted.

Overall, the findings reported here should stimulate more extensive clinical evaluation of the combination of tumor vaccines and MDX-CTLA-4. Although the preclinical experiments tested concurrent administration, this study illustrates that the temporal separation of immunization and antibody blockade

may also elicit important antitumor effects. Because our previous reports of GM-CSF-secreting melanoma vaccines similarly revealed the induction of a vasculopathy and of granulocyte, $CD4^+$, $CD8^+$, and $CD20^+$ lymphocyte infiltrates effectuating extensive tumor necrosis and fibrosis (21, 54), the current results suggest that MDX-CTLA-4 may amplify a long-lived memory response in patients (55, 56). Future comparison of the relative toxicity and immunogenicity of concurrent versus sequential combination therapy should provide a deeper understanding of the mechanisms limiting effective tumor immunity.

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Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma

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Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is a critical immunoregulatory molecule (expressed on activated T cells and a subset of regulatory T cells) capable of down-regulating T cell activation. Blockade of CTLA-4 has been shown in animal models to improve the effectiveness of cancer immunotherapy. We thus treated 14 patients with metastatic melanoma by using serial i.v. administration of a fully human anti-CTLA-4 antibody (MDX-010) in conjunction with s.c. vaccination with two modified HLA-A*0201-restricted peptides from the gp100 melanoma-associated antigen, gp100:209–217(210M) and gp100:280–288(288V). This blockade of CTLA-4 induced grade III/IV autoimmune manifestations in six patients (43%), including dermatitis, enterocolitis, hepatitis, and hypophysitis, and mediated objective cancer regression in three patients (21%; two complete and one partial responses). This study establishes CTLA-4 as an important molecule regulating tolerance to "self" antigens in humans and suggests a role for CTLA-4 blockade in breaking tolerance to human cancer antigens for cancer immunotherapy.

Optimal T cell activation requires interaction between the T cell receptor and specific antigen (1) (the first signal) and engagement of costimulatory receptors on the surface of the T cell with costimulatory ligands expressed by the antigen-presenting cell (APC) (the second signal). Failure of the T cell to receive a second signal can lead to clonal anergy (2). Two important T cell costimulatory receptors are CD28 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152) whose ligands on APC are B7-1 and B7-2 (3, 4). Although CD28 and CTLA-4 are closely related members of the Ig superfamily (5), they function antagonistically. CD28 is constitutively expressed on the surface of T cells (6), and upon engagement with B7-1 or B7-2, enhances the T cell receptor–peptide–MHC signal to promote T cell activation, proliferation, and IL-2 production (3, 7). CTLA-4 is not found on resting T cells but is up-regulated for 2–3 days after T cell activation (8, 9). CTLA-4 also binds to B7-1 and B7-2 but with greater affinity than CD28 (10) and antagonizes T cell activation, interferes with IL-2 production and IL-2 receptor expression, and interrupts cell cycle progression of activated T cells (11–14). The overall T cell response is determined by the integration of all signals, stimulatory and inhibitory.

Because CTLA-4 appears to undermine T cell activation, attempts have been made to block CTLA-4 activity in murine models of cancer immunotherapy. In mice implanted with immunogenic tumors, administration of anti-CTLA-4 Ab enhanced tumor rejection (15), although little effect was seen with poorly immunogenic tumors such as SM1 mammary carcinoma or B16 melanoma. Enhanced antitumor immunity was seen when anti-CTLA-4 Ab was given with granulocyte-macrophage colony-stimulating factor (GM-CSF)-transduced B16 cell vaccine and was associated with depigmentation, suggesting that at least part of the antitumor response was antigen-specific against "self" melanocyte differentiation antigens (16, 17). In a transgenic murine model of primary prostate cancer, administrating anti-CTLA-4 Ab plus GM-CSF-expressing prostate cancer cells reduced the incidence and histological severity of prostate cancer and led to prostatitis in normal mice, again suggesting an antigen-specific immune response against self-antigens in tumor rejection (18). Furthermore, because many human tumor antigens are normal self-antigens, breaking tolerance against self may be critical to the success of cancer immunotherapy.

Peptide vaccines against melanoma in humans can generate significant peptide- and tumor-specific reactivity, but clinical tumor regression was seen only very rarely unless IL-2 was administered concomitantly (19). The favorable tumor responses from CTLA-4 blockade in conjunction with tumor vaccines in murine models led to interest in using CTLA-4 blockade in human cancer immunotherapy. The production of a human mAb specific for blocking human CTLA-4 engagement to B7 has enabled us to evaluate the impact of CTLA-4 blockade in patients with metastatic melanoma receiving vaccinations with two HLA class I-restricted peptides from the gp100 melanoma-associated antigen, gp100:209–217(210M) and gp100:280–288(288V).

We report here on the development of significant clinical autoimmunity involving multiple normal human tissues and cancer regression in patients with metastatic melanoma receiving this treatment. This study establishes a clear role for CTLA-4 in the maintenance of peripheral tolerance in humans and suggests an important role for CTLA-4 in cancer immunotherapy and in the induction of human autoimmune diseases.

Methods

Patients and Treatment. All patients were HLA-A*0201$^+$ and had progressive stage IV melanoma, Karnofsky performance status ≥60%, and no evidence of autoimmune or immunodeficiency disease. Patients had never been immunized against gp100 and had no systemic therapy in the 3 weeks before treatment. All patients signed an informed consent and were treated in an approved protocol in the Surgery Branch at the National Institutes of Health. Patients underwent apheresis before treatment and 3 weeks after every two therapy cycles; peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque separation and cryopreserved at −180°C in heat-inactivated human AB serum with 10% DMSO. The trial used a two-stage

Abbreviations: CTLA-4, cytotoxic T lymphocyte-associated antigen 4; PBMC, peripheral blood mononuclear cells; APC, antigen-presenting cell.

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EXHIBIT B

Table 1. Patient characteristics, clinical response, and toxicity

Patient	Age/sex	Disease sites	Prior therapy	No. of cycles received*	Response (mos.)	Toxicity (grade III/IV)
1	52/M	Lung	I, S	2	PR (15+)	Enterocolitis; dermatitis
2	40/F	Supraclavicular lymph node	C, I, S	1	NR	Dermatitis; vitiligo†
3	39/M	Lung, mediastinum, subcutaneous	S	6	NR (mixed)	
4	55/F	Skin, subcutaneous	I, S	1	NR	Pulmonary infiltrates†
5	67/M	Liver, retroperitoneum, subcutaneous	C, I, R, S	4	NR	ANA+†
6	59/M	Lung, subcutaneous	I, S	4	NR	Vitiligo†
7	48/M	Lung, brain, adrenal, subcutaneous	I, S	2	NR	
8	48/M	Lung, liver, adrenal, mesentery, subcutaneous	C, I, S	2	NR	
9	53/M	Mediastinum, mesentery, skin	I, R, S	2	NR	Colitis
10	62/M	Lung, hilum	C, I, S	2	NR (mixed)	
11	54/M	Lung, brain, subcutaneous	C, S	5	CR (12+)	Hypophysitis
12	43/M	Subdiaphragm, muscle, subcutaneous	I, S	3	NR	Hepatitis; ANA+†
13	49/F	Lung, subcutaneous	C, I, S	4	CR (11+)	Dermatitis
14	63/M	Lung, pelvic lymph node	S	4	NR	

ANA, antinuclear Ab; C, chemotherapy; CR, complete response; F, female; I, immunotherapy; M, male; NR, no response; PR, partial response; R, radiotherapy; S, surgery.

*One treatment cycle consists of one infusion of anti-CTLA-4 Ab and one vaccination with gp100:209–217(210M) and gp100:280–288(288V) peptides.

†Grade I/II toxicity.

optimal design and was intended to accrue to 21 patients to the first stage. Because of the development of grade III/IV autoimmune toxicity in ≥3 patients, accrual ceased after 14 patients were enrolled.

Every 3 weeks, patients received anti-CTLA-4 Ab at 3 mg/kg i.v. over 90 min, followed by 1 mg of gp100:209–217(210M) peptide (IMDQVPFSV) emulsified in incomplete Freund's adjuvant (IFA) injected s.c. in one extremity and 1 mg of gp100:280–288(288V) peptide (YLEPGPVTV) emulsified in IFA injected in another extremity. The anti-CTLA-4 Ab (MDX-010; provided by Medarex) is a fully human $IgG_{1\kappa}$ Ab derived from transgenic mice having human genes encoding heavy and light chains to generate a functional human repertoire. This Ab has been shown to bind to CTLA-4 expressed on the surface of human T cells and inhibit binding of CTLA-4 to B7 molecules. Synthetic peptides were provided by the National Cancer Institute Cancer Therapy Evaluation Program.

Clinical Response Evaluation and Autoimmunity Screening. All patients underwent computed axial tomography of the chest, abdomen, and pelvis and MRI of the brain within 4 weeks of starting treatment and subsequently after every two therapy cycles. For each patient, the sum of the longest diameters of all tumors (World Health Organization Response Evaluation Criteria in Solid Tumors) before and after therapy was calculated. A partial response was defined as a decrease of ≥30% (but <100%) in the sum of the longest diameters of all evaluable metastases lasting ≥1 month with no new or enlarging tumors; a complete response was the disappearance of all evaluable tumors for ≥1 month. Patients not achieving either a partial or complete response were deemed nonresponders. To screen for autoimmunity, all patients before therapy received an opthalmologic examination that was repeated after 3 months on study. At baseline patients were negative for serum thyroglobulin Ab, rheumatoid factor, and antinuclear Ab; while on study, human anti-human (anti-idiotypic) Ab, erythrocyte sedimentation rate, antinuclear Ab, thyroid-stimulating hormone, and free T4 were tested every 3 weeks.

Pharmacokinetics. Plasma concentrations of MDX-010 were determined by ELISA using microtiter wells coated with CTLA-4-Ig (R & D Systems). Briefly, dilutions of plasma samples were incubated on the plates, and bound anti-CTLA-4 Ab was detected with alkaline phosphatase-labeled goat anti-human IgG F(ab)-specific probe, which was developed with *p*-nitrophenyl phosphate substrate.

Immunologic Assessment. For all assays, pretreatment and posttreatment cryopreserved PBMC samples were evaluated simultaneously.

EliSpot assay. PBMC were incubated for 24 h with peptide-pulsed C1R-A_2 APCs in 96-well plates coated with anti-IFN-γ Ab (BioSource, Camarillo, CA). The wells were then washed, coated with biotinylated anti-IFN-γ Ab (PharMingen), developed with avidin-alkaline phosphatase, and stained. The number of EliSpots per experiment was corrected by subtracting background spots caused by PBMC incubation with unpulsed APCs.

Flow cytometry and tetramer analysis. PBMC were Fc receptor-blocked with mouse IgG (Caltag, Burlingame, CA), stained with fluorochrome-labeled gp100:209–217:HLA-A*0201 tetramers (Beckman Coulter Immunomics, San Diego, CA) or Ab against selected T cell markers (BD Biosciences, San Diego), and analyzed by using a FACSCalibur with CELLQUEST (BD Biosciences).

In vitro sensitization assay. As described (19), PBMC were cultured in an Iscove's-based media with 10% heat-inactivated human AB serum with 1 μM native gp100:209–217 or gp100:280–288 peptide and 300 international units/ml of IL-2. After 11–13 days, these T cells were coincubated with peptide-pulsed T2 APC overnight and IFN-γ release in the supernatant measured by using ELISA. A positive assay is defined as IFN-γ release by posttherapy PBMC ≥100 pg/ml during incubation with a relevant peptide (gp100:209–217 or gp100:280–288), ≥2 times the IFN-γ released during incubation with an irrelevant control peptide, and ≥2 times the IFN-γ released by pretherapy samples.

Results

Patient Characteristics. All patients had stage IV melanoma (Table 1); 12 (86%) had visceral metastases. All had undergone excision of the primary lesion, and six patients (43%) had received prior chemotherapy. Eleven patients (79%) had received prior immunotherapy that included IFN-α (patients 2, 5–8, 10, 12, and 13), low-dose IL-2 (patients 2, 5, and 13), high-dose IL-2 (patients 4, 7, and 8), whole-cell melanoma vaccines (patients 1, 2, and 6), NY-ESO-1 peptide vaccine (patients 4 and 5), or granulocyte-macrophage colony-stimulating factor (patient 9).

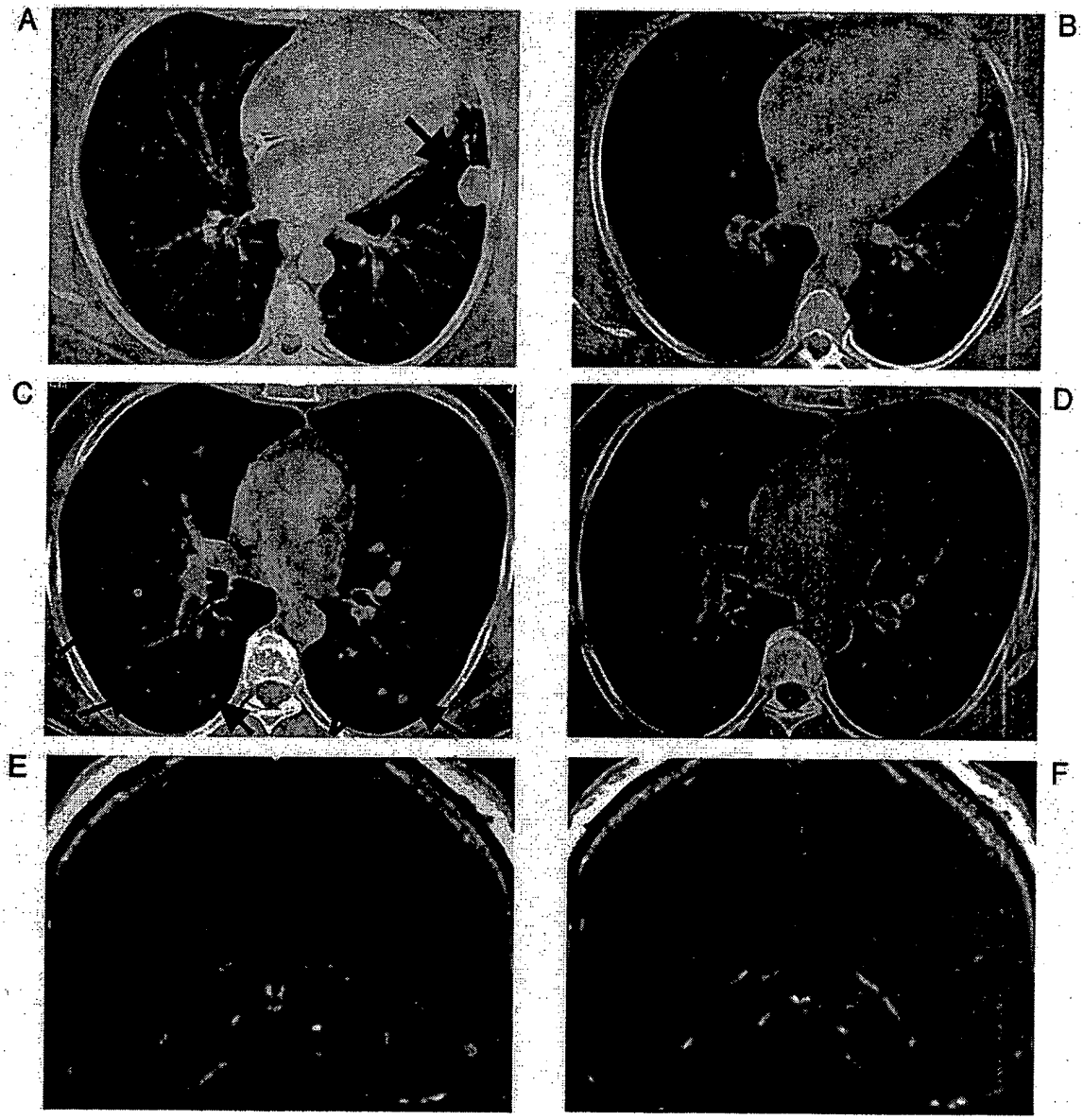

Fig. 1. Computed axial tomography scans illustrating pretherapy disease status (*Left*) and complete responses (*Right*) for patient 13 (*A* and *B*) and patient 11 (*C–F*). Arrows show sites of metastases.

Clinical Responses. Two patients experienced complete tumor responses, and one had a partial response (Table 1). Patient 1 had shrinkage of a solitary lung lesion after two treatment cycles. Patient 13 had complete resolution of a lung mass (Fig. 1 *A* and *B*) and a s.c. nodule after four cycles. Before therapy, patient 11 had 31 small lung nodules (Fig. 1*C*), two s.c. masses, and one 0.5-cm brain metastasis. The brain metastasis grew to ≈1 cm after two treatment cycles (Fig. 1*E*). Because of slight shrinkage in the s.c. nodules and some lung lesions, he received three additional cycles and subsequently had complete resolution of all metastases, including the brain lesion (Fig. 1 *D* and *F*). Two patients had mixed responses: patient 3 had disappearance of several lung nodules after four cycles but had progression of mediastinal lymph nodes; patient 10 had substantial shrinkage of a large hilar mass and several lung nodules after two cycles but had growth in other lung lesions.

Autoimmune Effects. Grade I/II adverse events included diarrhea (patients 3, 5, and 14), skin rash (patient 14), pulmonary infiltrates associated with mild pleuritic chest pain (patient 4), and vitiligo (patients 2 and 6 after one and two cycles, respectively). Autoimmune screening blood tests (see *Methods*) were normal except for patients 5 and 12 who developed antinuclear Ab after two and three cycles, respectively. Six patients (43%) developed seven grade III/IV autoimmune toxicities (Table 1), including three patients with dermatitis, two with colitis/enterocolitis, and one each with hypophysitis and hepatitis. Because autoimmunity in multiple organ sites induced by CTLA-4 blockade in humans has not been reported previously, the detailed histories of these patients are given below.

Patient 1 (a partial responder) developed a generalized erythematous maculopapular rash associated with severe pruritis 1 week after the second treatment. Skin biopsy showed perivascular lymphocytic and eosinophilic infiltrate, papillary dermal edema, and epidermal spongiosis consistent with an "allergic" drug eruption. Two days later, he developed severe diarrhea, requiring i.v. hydration. Upper and lower gastrointestinal en-

doscopy revealed multiple areas of inflammation and mucosal ulceration; duodenal and colonic biopsies showed marked lymphocytosis, plasmacytosis, and eosinophilia. Immunohistochemical studies showed predominance of $CD3^+$ cells ($CD8^+ > CD4^+$) in the inflammatory infiltrate, polyclonality of the plasma cells, and increased MHC-I and HLA-DR expression in the vasculature and epithelium. A diagnosis of autoimmune enterocolitis was made, and i.v. methylprednisolone was administered, resulting in marked clinical improvement within 24 h. The steroids were tapered over 5 days, and the patient had no further relapse of symptoms.

Patient 2 developed mild generalized pruritis 1 week after receiving her first treatment, which progressed over the next 2 weeks to a severe circumferential, erythematous, edematous macular rash on the extremities in which she had received her vaccine injections (right arm and left leg). Skin biopsy (Fig. 2*A*) showed epidermal spongiosis, extensive papillary dermal edema, and a prominent lymphocytic and eosinophilic infiltrate with vascular involvement as seen in collagen autoimmunity. Hydroxyzine and diphenhydramine gave symptomatic relief; the rash persisted for several weeks but slowly cleared. The patient developed vitiligo on both upper extremities over the next 3 weeks.

Patient 9 developed diarrhea 11 days after receiving his second treatment. Endoscopy showed pan-colitis. Colon biopsy (Fig. 2*B*) revealed severe inflammation with marked cellular infiltration and some crypt abscesses. Immunohistochemical studies demonstrated that the majority of infiltrating lymphocytes were $CD3^+$ (Fig. 2*C*) ($CD4^+ > CD8^+$, data not shown), plasma cells were polyclonal, and epithelial MHC-I and HLA-DR expression were increased (data not shown). The diarrhea was relieved upon treatment with i.v. methylprednisolone and was controlled with a slow taper of oral dexamethasone.

Patient 11 (a complete responder) developed progressive personality changes and memory problems soon after receiving his fourth and fifth cycles. MRI of the brain showed disappearance of a left temporal metastasis (Fig. 1 *E* and *F*); no other abnormalities were found. After the fifth treatment, further evaluation showed undetectable levels of thyroid-stimulating hormone, free T4, adrenocorticotropic hormone, cortisol, growth hormone, prolactin, and testosterone suggestive of panhypopituitarism. These same tests were in the normal range in cryopreserved pretherapy plasma samples. Focused MRI indicated the size of the pituitary gland to be at the upper limit of normal. Because the patient had a complete clinical response (resolution of a brain lesion, two s.c. nodules, and 31 lung metastases), we were reluctant to administer high-dose immunosuppressive steroids to treat the hypophysitis. Subsequently the patient received replacement doses of thyroxine, testosterone, and hydrocortisone, and his personality and memory abnormalities were resolved. Follow-up pituitary MRI done 6 weeks later showed a slight decrease in size, perhaps reflecting decreased inflammation in the gland.

Patient 12 developed abnormal liver enzymes and antinuclear Ab on routine blood tests done 3 weeks after his third treatment. Other serum tests for autoimmunity were unremarkable. Liver biopsy (Fig. 2*D*) revealed acute hepatitis with numerous foci of lobular inflammation consisting mainly of lymphocytes with some plasmacytes and eosinophils, consistent with autoimmune processes. Immunohistochemical studies showed a predominantly $CD3^+$ cellular infiltrate with $CD4^+$ cells mainly in the peri-portal areas and $CD8^+$ cells mainly in the hepatic lobules. Over the next 2 weeks his alanine aminotransferase levels peaked at 2,860 units/liter (normal, 6–41), and aspartate aminotransferase levels peaked at 1,193 units/liter (normal, 9–34). Low-dose oral prednisone therapy was initiated, and all values decreased slowly to normal over the next 4 months.

Patient 13 (a complete responder) developed a severe gener-

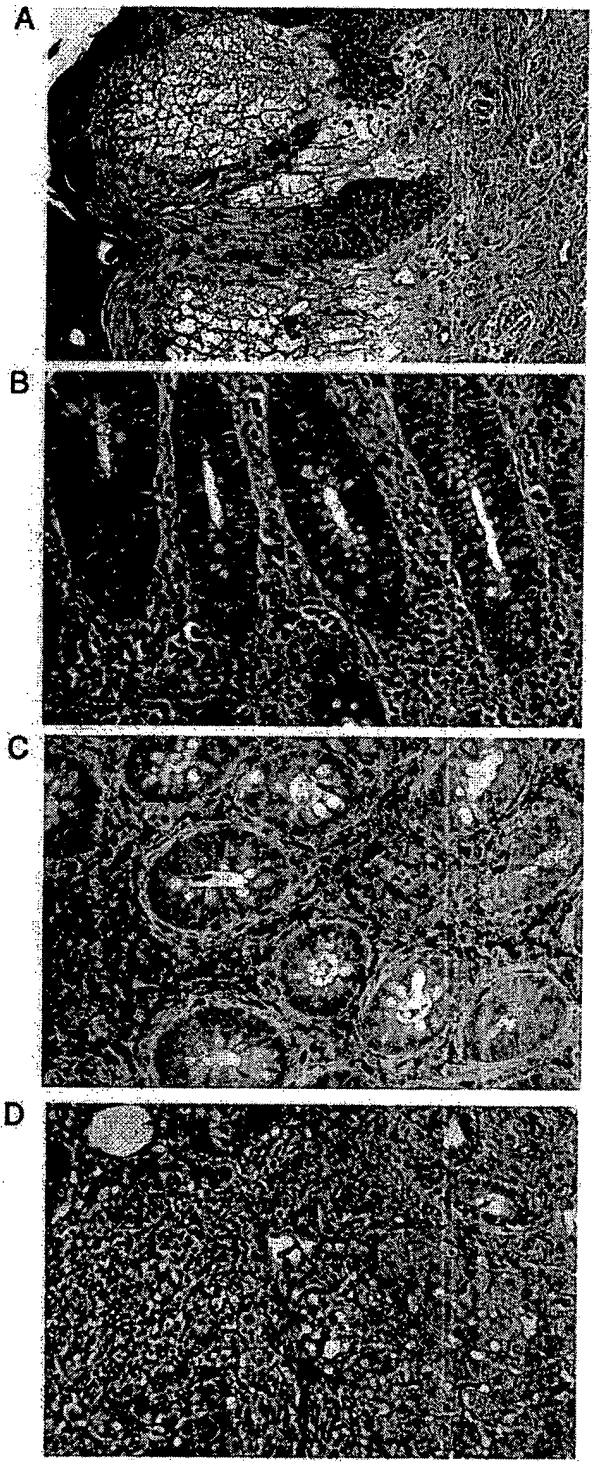

Fig. 2. Histopathologic analyses of selected patients experiencing autoimmune events. (*A*) Skin biopsy (patient 2) showing severe dermatitis with epidermal spongiosis, papillary dermal edema, and a prominent inflammatory infiltrate in both the superficial and deep dermis. (*B*) Colon biopsy (patient 9) illustrating severe colitis with infiltration of the lamina propria with neutrophils, lymphocytes, monocytes, plasmacytes, and eosinophils suggestive of autoimmune reactions. Neutrophils and lymphocytes also infiltrate the crypts; numerous mitotic figures are seen in the epithelial cells lining the crypts. (*C*) Immunohistochemistry of the colon biopsy (patient 9) reveals that the majority of cells are $CD3^+$. (*D*) Liver biopsy (patient 12) showing areas of necrosis and lobular inflammation with (mainly) lymphocytic infiltration into the portal triad. (Magnifications: *A*, ×10; *B–D*, ×20.)

Table 2. Flow cytometric analysis of selected T cell surface markers before and after two treatment cycles

	HLA-DR$^+$		CD45RO$^+$		CD69$^+$		CD4$^+$CD25$^+$		CD25$^+$		CTLA-4$^+$	
Patient	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
	% of CD3$^+$CD4$^+$		% of CD3$^+$CD4$^+$		% of CD3$^+$CD4$^+$		% of CD3$^+$		% of CD4$^+$		% of CD4$^+$CD25$^+$	
1	17.5	41.8	85.4	88.9	4.6	4.3	15.9	11.4	41.8	35.0	0.4	2.5
3	5.2	17.3	78.8	82.2	1.7	3.4	19.5	10.7	34.6	26.7	3.0	1.1
5	17.6	29.2	77.2	84.6	2.8	1.5	19.0	18.2	42.3	36.4	2.0	1.1
6	31.3	42.5	98.9	99.0	4.4	3.1	14.3	13.5	46.3	35.8	1.5	0.6
7	6.2	20.3	57.0	72.9	1.4	2.0	14.1	23.8	26.7	40.8	0.4	2.4
10	9.8	22.7	85.7	94.3	2.4	3.6	23.4	19.4	46.5	35.5	0.2	1.7
11	6.2	12.2	95.2	89.6	9.8	1.7	21.2	17.6	43.3	25.4	0.0	0.6
12	3.9	31.7	73.7	76.4	1.5	3.9	10.1	11.1	19.9	26.0	0.2	5.5
14	7.9	39.4	72.4	89.4	2.3	6.1	12.8	7.0	28.9	20.5	0.6	2.5
Mean Δ ± SEM	+16.8 ± 2.9 (P = 0.0004)		+5.9 ± 2.4 (P = 0.04)		−0.1 ± 1.1 (P = 0.9)		−1.9 ± 1.8 (P = 0.3)		−5.4 ± 3.2 (P = 0.13)		+1.1 ± 0.7 (P = 0.17)	
	% of CD3$^+$CD4$^-$		% of CD3$^+$CD4$^-$		% of CD3$^+$CD4$^-$							
1	30.5	57.9	66.1	69.0	7.7	6.8						
3	14.8	22.3	52.4	58.8	3.6	3.9						
5	28.9	31.3	53.8	55.8	4.3	2.7						
6	78.0	67.4	51.3	61.4	12.5	8.2						
7	12.3	13.3	52.9	43.7	6.7	2.5						
10	29.6	44.3	80.2	82.0	2.1	7.8						
11	4.9	18.2	77.6	70.4	8.1	7.4						
12	8.4	35.8	69.6	72.1	3.8	8.9						
14	14.1	20.7	57.9	60.9	7.0	7.6						
Mean Δ ± SEM	+10.0 ± 4.1 (P = 0.04)		+1.4 ± 2.0 (P = 0.51)		0.0 ± 1.2 (P = 1.0)							

Values compared by using paired t test.

alized erythematous and pruritic rash 1 week after receiving her fourth treatment. Biopsy showed perivascular lymphocytic infiltration with abundant eosinophils in the superficial dermis; immunohistochemical studies revealed that the lymphocytes were mainly CD3$^+$ cells (CD4$^+$ > CD8$^+$). Lymphocytes cultured from a biopsy of the rash were all CD8$^+$, and 97% reacted with gp100:209–217:HLA-A*0201 tetramer (data not shown). The rash resolved slowly upon treatment with hydroxyzine.

Pharmacokinetics. The mean peak of MDX-010 after the first dose was 72 ± 33 μg/ml, and the trough before the second dose was 12 ± 7 μg/ml. Modestly cumulative levels were seen in most patients with repeated dosing. The mean plasma level posttherapy (counting all cycles) was 99 ± 41 μg/ml, which decreased to 17 ± 10 μg/ml 3 weeks later. No clear correlation between plasma concentrations or Ab clearance and tumor regression or toxicity was seen.

Immunologic Response. Using the EliSpot assay to quantify the frequency of cells reactive (secreting IFN-γ) to native gp100:209–217 and gp100:280–288 peptides, PBMC from 11 patients after one to six treatment cycles generated <10 (corrected) EliSpots per 10^5 cells, which is below the limit of detection for appropriate assessment of immunization (data not shown). To quantify the frequency of cells capable of recognizing the gp100:209–217:HLA-A*0201 complex, tetramer analysis was performed on PBMC from seven patients after one to four cycles and failed to show a difference between pretherapy and posttherapy samples in binding to gp100:209–217:HLA-A*0201, although <1% of CD8$^+$ cells were tetramer positive in all samples (data not shown).

Using an *in vitro* sensitization (IVS) assay, which is more sensitive than the EliSpot or tetramer assays to identify the presence of specific immunologic reactivity, all 11 patients with PBMC available for testing developed specific immunity against the native gp100:209–217 peptide after one to four treatment cycles compared with six patients (55%) who developed specific immunity against the native gp100:280–288 peptide (data not shown). The results of the EliSpot, tetramer, and IVS assays are consistent with our previous experiences with peptide vaccination alone (19–21) and provide no indication that simultaneous administration of anti-CTLA-4 Ab enhanced responses against the immunizing peptides.

Using flow cytometry to compare surface marker expression on PBMC of nine patients before and after two treatment cycles (Table 2), HLA-DR expression (an activation marker) was significantly increased on posttherapy CD3$^+$CD4$^+$ cells (P = 0.0004; paired t test) and CD3$^+$CD4$^-$ (presumably CD8$^+$) cells (P = 0.04). CD3$^+$CD4$^+$ cells also showed significantly increased expression of CD45RO (a memory cell marker) posttherapy (P = 0.04). The percent of various cell populations expressing CD69, CD25, and CTLA-4 did not change.

Discussion

An understanding of the mechanisms that underlie the ability of the immune system to respond appropriately to "foreign" antigens and yet not react to self-antigens is a fundamental goal of studies in immunology. Although clonal deletion mechanisms may be important in eliminating lymphocyte precursors that bind with high avidity to self-antigens, many alternative mechanisms for inducing peripheral tolerance exist. Immunomodulatory cytokines may play a role in these alternative processes: transforming growth factor-β has been shown to inhibit T cell activation and proliferation (22) and induce FasL expression, leading to activation-induced cell death (23); IL-10 can negatively affect dendritic cell maturation (24), leading to APC apoptosis (25). Depletion of essential nutrients, such as tryptophan or cysteine, from the lymphocyte microenvironment may also contribute to self-tolerance (26, 27). Killer inhibitory receptors expressed by natural killer cells, as well as some T cells, prevent activation against noninfected host cells. Furthermore, a subset of CD4$^+$CD25$^+$ T cells with immunoregulatory capabilities has been shown to prevent T cell activation and proliferation (28–30). Although the exact mechanism of CD4$^+$CD25$^+$-associated suppression is uncertain, the ubiqui-

tous presence of CTLA-4 on these regulatory T cells (31, 32) suggests that this molecule may be involved.

A negative immunoregulatory role of CTLA-4 was definitively demonstrated in CTLA-4-deficient mice that developed lethal lymphoproliferative disease with infiltration of visceral organs by activated T cell blasts (33). In animal models, blockade of CTLA-4 has led to enhanced antitumor immunity (15–18) and intensification of T cell-associated autoimmune encephalomyelitis, colitis, and diabetes (31, 34, 35). Thus before clinical use, MDX-010 anti-CTLA-4 Ab underwent extensive evaluation in cynomologus monkeys and did not cause any notable clinical or pathological toxicity at repeated i.v. doses from 3 mg/kg to 30 mg/kg in acute and chronic toxicology studies (unpublished data from Medarex).

CTLA-4 blockade in humans given a one-time 3 mg/kg dose of MDX-010 to seven patients with metastatic melanoma and two with ovarian carcinoma caused no significant toxicity, although all seven melanoma patients developed an asymptomatic grade I rash with skin biopsies showing peri-vascular T cell infiltrates (36). Although objective cancer regressions were not shown, three melanoma patients had evidence of tumor necrosis at biopsied sites.

The study presented here involved anti-CTLA-4 Ab given repeatedly along with tumor-specific peptide vaccinations. Significant grade III/IV autoimmunity in several organ systems was frequent (43%; 6 patients of 14), thus indicating an important role for CTLA-4 in the maintenance of peripheral tolerance to self-antigens. It is unlikely that the peptide immunizations played a role in inducing autoimmunity in the colon, liver, and pituitary because the target antigens are not thought to be expressed in these tissues and because these autoimmune events were not seen in prior clinical trials using the same vaccines (19–21). Importantly, after discontinuation of further treatment cycles and given supportive care and/or steroid therapy, all patients experiencing autoimmunity recovered from the acute toxicity phase and did not relapse or develop subsequent autoimmune events as the Ab levels decreased over time.

Notably, three patients experienced objective cancer regression, and two experienced mixed responses. In our prior experience with patients receiving one or two HLA class I-restricted peptides alone, no objective cancer regressions were seen in 33 patients (19, 21). Clinical responses were seen only when IL-2 was administered concomitantly. Thus it is very likely that the anti-CTLA-4 Ab was involved in mediating cancer regression in the patients described in the current series. The role of the peptide vaccines given with the anti-CTLA-4 Ab in the cancer regression is uncertain. All patients developed T cell reactivity against the immunizing peptides given in conjunction with the anti-CTLA-4 Ab. The levels of T cell reactivity were consistent with our prior clinical trials using the same peptides without CTLA-4 blockade (19–21). The fact that all three patients with objective responses developed grade III/IV autoimmunity and that two additional patients developed vitiligo suggests that the anticancer response may be associated with some degree of heightened reactivity against self-antigens as well.

Evaluation of lymphocytes from the peripheral circulation showed a significant increase in activation markers after just two cycles of therapy. The increased expression of HLA-DR and CD45RO was especially apparent in the $CD3^+CD4^+$ subset (despite the patients having been vaccinated with two class I-restricted peptides aimed at immunizing $CD8^+$ cells), consistent with the $CD4^+$ T cell-driven lymphoproliferative syndrome observed in CTLA-4-deficient mice (33). We did not find a depletion of CTLA-4-expressing cells or $CD4^+CD25^+$ regulatory T cells posttherapy. However, these measurements and all tests of immunologic reactivity were conducted by using peripheral blood lymphocytes, which might not reflect the immune status of the tumor microenvironment (37).

This clinical trial has indicated a potential role for CTLA-4 blockade in cancer immunotherapy and has established an important role for CTLA-4 in the maintenance of peripheral tolerance against self antigens in humans. Further study with MDX-010 anti-CTLA-4 Ab either alone or in conjunction with vaccines will be useful to elucidate the potential relationship between autoimmune effects against normal tissues and against tumors and the possible means to dissociate these two events.

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